Institut für Veterinärphysiologie
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Arbeit unter wissenschaftlicher Betreuung von
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Ehlers-Danlos syndrome vascular type: Mouse model-based read-out system to assess drug effects on the mechanical property of the thoracic aorta

Inaugural-Dissertation

zur Erlangung der Doktorwürde der
Vetsuisse-Fakultät Universität Zürich

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2016

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Summary
Ehlers-Danlos syndrome vascular type (EDS IV) is an autosomal dominant connective tissue disorder caused by *COL3A1* mutations. Recently, a novel mouse model with *Col3a1* haploinsufficiency has been described in which heterozygotes have an increased risk for aortic rupture, comparable to the human phenotype. The goals of the study were (1) to obtain a reliable read-out for the difference in the mechanical integrity of the thoracic aorta between heterozygotes and wild types as well as (2) to assess the effect of a candidate drug on aortic tissue. 1.5-mm-width rings of the thoracic aorta were uniaxially stretched until rupture. Tensile force at tissue rupture was significantly lower in heterozygotes compared to wild types and decreased with increasing section distance from the heart. In samples from heterozygotes, electron microscopy showed higher variability in the diameters of collagen fibrils and multiphoton microscopy indicated reduced collagen amount and density. After treatment between 4 and 8 weeks of age with doxycycline, rupture force of thoracic aortic wall from heterozygotes increased to the level of wild types. This system is suitable for detecting significant differences in the rupture force of the thoracic aorta between wild-type and *Col3a1* haploinsufficient mice and allows testing candidate substances that may increase the mechanical stability of the aorta. This study confirms the benefit of doxycycline in the thoracic aorta of mice with *Col3a1* haploinsufficiency.

Keywords: aorta, mouse, Ehlers-Danlos syndrome vascular type (EDS IV), doxycycline

Zusammenfassung
Ehlers-Danlos Syndrom vaskulärer Typ (EDS IV) ist eine autosomal dominant vererbte Bindegewebskrankheit, die durch *COL3A1*-Mutationen verursacht wird. Vergleichbar zum humanen Phänotyp zeigt ein kürzlich beschriebenes Mausmodell mit echter Haploinsuffizienz von *Col3a1* eine erhöhte Mortalität infolge spontaner Rupturen der Aorta. Ziele dieser Studie waren (1) ein entsprechendes Messsystem zu finden, welches den Unterschied in der mechanischen Stabilität der thorakalen Aorta in diesem Mausmodell im Vergleich zu Wildtyp-Mäusen erfassen kann, wie auch (2) die Wirkung einer viel versprechenden Substanz auf Aortengewebe zu bestimmen. 1,5 mm breite Ringe der thorakalen Aorta wurden bis zum Geweberiss gestreckt, wobei die maximale Zugkraft (mN) gemessen wurde. Die maximale Zugkraft bei Heterozygoten war signifikant niedriger im Vergleich zu den Wildtyp-Mäusen. Die mechanische Stabilität der Aorta nahm bei beiden Genotypen mit zunehmendem Abstand vom Herz ab. Das etablierte Messsystem ist geeignet, den Unterschied in der Stabilität der Mausaortenwand zwischen wildtyp und heterozygoten Tieren zu bestimmen und dadurch Arzneistoffe für die Erhöhung der mechanischen Stabilität der Aorta zu testen. Diese Studie bestätigt einen positiven Einfluss von Doxyzyklin auf die mechanische Eigenschaft der thorakalen Aorta in Mausmodellen mit *Col3a1*-Haploinsuffizienz.

Schlüsselwörter: Aorta, Maus, Ehlers-Danlos Syndrom vaskulärer Typ (EDS IV), Doxyzyklin

Cardiovascular Drugs and Therapy – Original Article

Ehlers-Danlos syndrome vascular type: Mouse model-based read-out system to assess drug effects on the mechanical property of the thoracic aorta

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Word count: 3378

Acknowledgements
We are grateful to Dr Arabella Mauri and Prof Dr Edoardo Mazza for help in biomechanics, multiphoton microscopy, and custom-made device development (manufacturing work by Jean-Claude Tomasina) as well as for comments on the manuscript. We also thank Carmen Kaiser and Gery Barmettler for help with nonlinear laser scanning and electron microscopy as well as Prof Dr Max Gassmann for helpful discussions. We also thank Prof Dr Edouard Battegay, Dr Indranil Bhattacharya, Julien Courseau, Dr Cecilia Giunta, Dr Stephan Gysi, Dr Elvira Haas, Dr Raimund Jaeger, Prof Dr Beat Steinmann, and Judith de Vos for support.

This work was supported by grants from the Gebauer Stiftung, Isaac Dreyfus-Bernheim Stiftung, Parrotia-Stiftung, Sutter-Stöttner Stiftung, and Wolfermann-Nägeli-Stiftung.

Abstract

Purpose Ehlers-Danlos syndrome vascular type (EDS IV) is an autosomal dominant connective tissue disorder caused by *COL3A1* mutations. Recently, a novel mouse model with *Col3a1* haploinsufficiency has been described in which heterozygotes have an increased risk for aortic rupture, comparable to the human phenotype. Our goals were to obtain a reliable read-out for the clinically relevant difference in the mechanical integrity of the thoracic aorta between heterozygous and wild-type mice as well as to assess the effect of a candidate drug on aortic tissue as a proof-of-principle test.

Methods and Results 1.5-mm-width rings of the thoracic aorta were uniaxially stretched until rupture. Tensile force at tissue rupture was significantly lower in heterozygotes compared to wild types and decreased with increasing section distance from the heart. In samples from heterozygotes, electron microscopy showed higher variability in the diameters of collagen fibrils and multiphoton microscopy indicated reduced collagen amount and density, explaining the decrease in rupture force. After treatment between 4 and 8 weeks of age with the matrix metalloproteinase inhibitor doxycycline, our proof-of-principle test demonstrated that the rupture force of thoracic aortic wall from heterozygotes increased to the level of wild types.

Conclusions Our read-out system is suitable for detecting significant differences in the rupture force of the thoracic aorta between wild-type and *Col3a1* haploinsufficient mice. This system allows testing candidate substances that may increase the mechanical stability of the aorta. Our data confirm the benefit of doxycycline in the thoracic aorta of mice with *Col3a1* haploinsufficiency.

Keywords *Col3a1*, Ehlers-Danlos syndrome vascular type (EDS IV), mechanical integrity, aorta, mouse model, doxycycline

Introduction

Ehlers-Danlos syndrome vascular type (EDS IV) is an autosomal dominant connective tissue disorder with a prevalence of ~2 in 100‘000 individuals [1]. It is characterized by thin translucent skin, easy bruising, typical facial features, and fragility of hollow organ walls, including larger arteries, which increases risk of tissue rupture [2]. The most severe complication is therefore the increased risk for dissection and rupture of the aorta and other large arteries, which may lead to sudden death without warning at normal size. So far, only disease management, including medication with the antihypertensive drug celiprolol, and treatment of symptoms have been implemented but there is no targeted therapy available [3-5].

EDS IV is caused by mutations in the gene *COL3A1*, which encodes the alpha 1 chain of type III collagen, a fibrillar collagen [6, 7]. Type III collagen is abundantly expressed in arterial walls, within which it is integrated into the collagenous network of the adventitia and the elastin lamellae of the media (www.gtexportal.org/home/gene/COL3A1). In the majority of EDS IV cases, missense mutations leading to glycine substitution have been reported (cf. HGMD Professional and LOVD). Less frequently, other missense or splice site mutations or small deletions/insertions have also been detected. Only few nonsense mutations leading to functional *COL3A1* haploinsufficiency and one case of true *COL3A1* haploinsufficiency have been described so far (cf. in haploinsufficiency a gene has only a single functional copy instead of two copies) [8-10].

For EDS IV, only two mouse models are available, each of which with suspected true *Col3a1* haploinsufficiency. Heterozygous animals of the first mouse model (C.129S4(B6)-Col3a1tm1Jae/J) presented only a weakly pronounced phenotype and no increased mortality rate [11]. The described phenotypic features of heterozygous animals include reduced collagen content in the arterial walls, spontaneous development of lesions of various severity in the aorta, and reduced abdominal aortic wall strength at the age of 21 months [12]. Previous studies showed that treatment with the broad-spectrum matrix metalloproteinase (MMP) inhibitor doxycycline protected 9-month-old heterozygous mice from histologically identifiable aortic lesions and normalized aortic collagen content [13, 14].

The second, more recent, mouse model with a *Col3a1* mutation (Col3 alpha 1 delta) results in an increased mortality rate due to spontaneous rupture of the thoracic aorta in ~28% of heterozygous mice [15]. This model has a much more pronounced cardiovascular phenotype, which corresponds more to the phenotype of EDS IV patients. So far, however, neither the mechanical integrity of the aorta nor therapeutic approaches have been tested in this mouse model.

Our goals were to develop a reliable read-out system for the assessment of the clinically relevant mechanical integrity of the thoracic aorta as well as to determine whether in the second mouse model (Col3 alpha 1 delta) the expected reduced mechanical integrity of the aortic wall can be measured and hence objectively characterized. To provide a proof of principle for our read-out system, we treated these mice with doxycycline, thereby showing that the therapeutic effect of doxycycline derived from the first mouse model can be reproduced in the second *Col3a1* mouse model.

Materials and Methods

Animals

Mice with a heterozygous mutation leading to true *Col3a1* haploinsufficiency (Col3 alpha 1 delta) [15] were obtained from MRC Harwell Laboratories (Oxfordshire, UK) and bred in a vivarium on a 12-hour light/dark cycle receiving standard rodent chow and water *ad libitum*.

Due to mixed genetic background (C57BL/6J and 129P/OlaHsd) mice were backcrossed with the C57BL/6J strain, before starting the measurements. *Col3a1* genotype was determined after weaning by PCR with genomic DNA of ear biopsies using following primers: 5′-TTGGCAAATCCTAAATAACTTC-3′ (forward, specific to the wild-type allele), 5′-GTCCCTGGATTGCTTAGTCTTA-3′ (forward, specific to the mutant allele), and 5′-GTTGGCTTGCTTGTGTATATATG-3′ (reverse). All animal experiments were performed in accordance to Swiss federal animal regulations and were approved by the local authorities. The experiments conform to the latest (2011) Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (www.nap.edu).

Measurement of the mechanical integrity of the aorta

For the assessment of differences in the mechanical integrity of the thoracic aorta between heterozygotes and wild types, groups of genotype-, age-, and sex-matched mice were analyzed at 9-11 weeks, 6-7 weeks, and 4-5 weeks of age. Whereas in the groups of 6-7- and 9-11-week-old animals each five heterozygous and wild-type mice per sex were examined, in the group of 4-5-week-old mice only five males per genotype were available.

Immediately following sacrifice using carbon dioxide, the thoracic aorta was isolated, excised, and carefully cleaned of adherent connective tissue and fat in MOPS (3-(N-morpholino)-propanesulfonic acid) buffer. 1.5-mm sections (S1-S3) were cut from the ascending and descending aorta (Fig. 1a) by a custom-made device (Supplementary Fig. 1a) and mounted on two 200-µm-diameter stainless steel wires (Supplementary Fig. 1b, c) placed in MOPS buffer. The mounted aortic rings were stretched with constant speed of 5 µm/s until tissue rupture using a uniaxial tension device (Tissue Puller 560TP, Danish Myo Technology, Aarhus, Denmark), thereby recording the maximum stretching/tensile force in mN.

Transmission electron microscopy

Samples from the thoracic aorta and skin of two wild-type and two heterozygous mice (each 16 weeks of age) were fixed over night with 2.5% glutaraldehyde in Na-cacodylate buffer (0.1 M, pH 7.35), rinsed in Na-cacodylate buffer, and postfixed with 1% OsO4 in Na-cacodylate buffer for one hour on ice. Subsequently, samples were rinsed in sterile endotoxin-free water and *en bloc* stained with 1% aqueous uranyl acetate. Samples were dehydrated in graded ethanol as well as propyleneoxide and subsequently embedded in Epon-Araldite (Sigma-Aldrich, St. Louis, MO, USA). Thin sections (70 nm) were cut on a Reichert-Jung Ultracut E (Leica Microsystems, Heerbrugg, Switzerland), stained with uranyl acetate and lead citrate, and examined under a FEI CM100 transmission electron microscope (FEI, Eindhoven, The Netherlands).

In the images of the adventitia of the thoracic aorta of one wild-type and one heterozygous animal, a 2 µm × 2 µm area was arbitrary chosen and therein fibrils were counted and measured for diameters using Fiji (Open Source) software (http://fiji.sc/Fiji).

Multiphoton microscopy

For three wild-type and three heterozygous mice (each 11 weeks of age), the collagen microstructure of aortic tissue under stretching was investigated with a dedicated *in situ* experimental setup [16, 17]. Briefly, mounting pins were integrated on a custom-made stretching device, which was placed under a multiphoton microscope (Fluoview 1000 MPE, Olympus). The stretching device consists of an actuator driven by a servo motor and two counter-rotating spindles, allowing for a controlled and symmetrical displacement of the two pins at a stretching velocity of 0.02 µm/s. Imaging was performed on the sample submerged in saline solution (0.9% NaCl) with an immersion objective (LUMPLFLN 40× W, NA 0.8). Sections from the thoracic aorta were stained with a DNA-binding dye (Hoechst 3334210)

before imaging with an excitation wavelength of 820 nm. The second harmonic generation (SHG) signal emitted by collagen was detected with a specific filter (Olympus FV10-MRROPT, BA397-412) separately from the fluorescence of the cell nuclei and elastin (BA455-490). 3-D stacks were taken at unstretched configuration and at three stretching steps corresponding to a displacement (λ) of 1.9 mm, 2.6 mm, and 3.2 mm.

Microstructural parameters were quantified from microscopy images containing only SHG signal using custom scripts in Matlab (The MathWorks Inc., MA, USA). Collagen amount and density were quantified after converting grayscale images to black and white images with an adequate threshold of 0.1, so that white pixels represent collagen. Collagen amount was extracted by the computed white voxels. Density coefficient was defined as the fraction of white pixels to the total amount of pixels in the image and mean density coefficient was estimated by averaging the density coefficients over all images in the collagenous layer of the stack.

Doxycycline treatment

A group of heterozygous animals was treated for 4 weeks with doxycycline (800 mg/kg in food from Kliba Nafag, Kaiseraugst, Switzerland) starting at 4 weeks of age. Control groups of wild-type and heterozygous mice remained untreated and were fed with control diet (3802.PX.F12 mouse/rat diet). Initially, each group consisted of 16 animals (8 males and 8 females). During the 4-week experiment, a total of 12 animals died. All but one were heterozygous (4 untreated and 7 treated) and died due to spontaneous thoracic aortic rupture within the first 2 weeks of the experiment. After 4 weeks, survived mice (15 wild types, 12 untreated and 9 treated heterozygotes) were euthanized and for thoracic aortic ring sections (S1-S3) the tensile force necessary to induce tissue rupture was measured by an examiner (J.Mü.) blinded to both genotype and treatment. Aortic rings from 12 wild types, 9 untreated and 9 treated heterozygotes were available for successful tensile force measurements.

Statistical analysis

If not stated otherwise, for arithmetic means upper and lower confidence limits (95% confidence intervals, CI) were calculated using critical values of paired *t*-test distribution (P=0.05) and indicated where appropriate.

Results

Mechanical integrity of the thoracic aorta

The mechanical integrity of the thoracic aorta was assessed by measuring the tensile force required to rupture 1.5-mm-width aortic ring sections (S1-S3, Fig. 1a). In wild-type mice, these rupture forces were significantly higher for the ascending aorta (S1) compared to the descending part (S2 and S3) regardless of age (Fig. 1b and Supplementary Table 1). In heterozygous mice, the difference between rupture forces of the ascending and the descending aortic sections was less pronounced (Fig. 1c and Supplementary Table 1). More importantly, the rupture forces from heterozygotes were significantly lower compared to wild types in all three aortic sections from the age of 6-7 weeks (Fig. 1c and Supplementary Table 1). Between male and female animals, there were no considerable differences in rupture forces (Supplementary Fig. 2 and Supplementary Table 1).

Transmission electron microscopy
Electron microscopy revealed reduced density and more variable diameters of collagen fibrils in the adventitia of the thoracic aorta from heterozygous compared to wild-type mice (Fig. 2a, b). A disorganized pattern of collagen fibrils was also observed in the skin samples of heterozygous mice (Fig. 2c, d). The reduced density of collagen fibrils and the higher variability of fibril diameters with more oversized fibrils in Col3a1 deficiency were objectified by counting collagen fibrils and by measuring fibril diameters in 2 µm × 2 µm image areas. We found that the number of fibrils in the adventitia of one arbitrary selected heterozygote (216 fibrils) was reduced to approximately one-half of that of one arbitrary selected wild type (455 fibrils), whereas the mean diameter of the collagen fibrils in the heterozygote was significantly higher than that of the wild type (Supplementary Fig. 3).

Multiphoton microscopy
Multiphoton microscopy images of aortic wall samples in unstretched configuration revealed considerably less collagen in heterozygous compared to wild-type mice (Fig. 3a and Supplementary Table 2). The density coefficient was also lower in samples of heterozygous mice, indicating larger spaces in the collagen network (Supplementary Table 2). Analysis of the collagen network during stretching revealed further differences between samples from heterozygous and wild-type mice (Fig. 3b). While in the aortic tissue from wild-type animals the collagen structure started reorienting and aligning at the first stretching step (displacement of 1.9 mm), that of heterozygotes showed little reaction until the last stretching step (displacement of 3.2 mm).

Doxycycline treatment
As a proof-of-principle, we tested the effect of the MMP inhibitor doxycycline using our read-out system. In heterozygous mice treated with doxycycline, the tensile forces at tissue rupture were comparable to wild-type levels, confirming the positive effect of doxycycline on the mechanical integrity of the thoracic aorta in our mouse model (Fig. 4 and Supplementary Table 3). During the study, all mice gained weight according to their age; at the beginning and the end of the treatment there was no significant difference in body weights among wild-type and heterozygous mice untreated or treated with doxycycline (Supplementary Fig. 4).

Discussion

In this study, we describe a read-out system for the assessment of the mechanical integrity of mouse thoracic aorta and provide hitherto unprecedented evidence to be considered in haploinsufficiency of type III collagen. The choice and mechanical characterization of the thoracic part of the aorta is based on the clinical context of EDS IV pathology such as aortic dissection and rupture with or without prior mild dilatation or aneurysm formation.

Our read-out system revealed significant rupture force differences between wild-type and heterozygous animals of the novel (second) *Col3a1* mouse model (Col3 alpha 1 delta backcrossed to C57BL/6J) from the age of few weeks (Fig. 1c). In contrast, for the first available *Col3a1* mouse model (C.129S4(B6)-Col3a1tm1Jae/J maintained on a BALB/c background) a significant difference in the mechanical integrity of abdominal aorta was only detectable at the age of 21 months using a comparable read-out system [12]. There may be multiple reasons for this discrepancy between the two available *Col3a1* mouse models. It could be due to methodological differences as different parts of the aorta were analyzed (thoracic vs. abdominal) or due to differences in the mouse models such as different *Col3a1* mutations and genetic backgrounds (C57BL/6J vs. BALB/c). In further studies, analysis of the first mouse model (C.129S4(B6)-Col3a1tm1Jae/J) by using our read-out system as well as

backcrossing the mouse model used in this study (Col3 alpha 1 delta) to its second background (129P2/OlaHsd) [15] may help to elucidate this discrepancy.

By using our read-out system, the maximum tensile force needed to induce rupture of the aortic wall was not only significantly lower in heterozygous mice but also significantly decreased with increasing distance from the heart (Fig. 1b). Indeed, in wild-type mice the forces breaking 1.5-mm-width rings of the ascending aorta were significantly higher than that of the descending aorta. Although extensively studied [18-20], to the best of our knowledge, this has not been previously reported for mice. For pig, which currently provides the most suitable animal model of the human aorta, however, mechanical properties of different segments of the thoracic aorta were assessed by measuring tensile strengths (maximum stress) [21]. Considering that tensile strength (N/m^2) is defined as tensile force (N) per unstretched cross-sectional area (m^2) and that the aortic wall thickness (area) gradually decreases down the thoracic aorta [18-21], the longitudinally increasing stress at rupture of the porcine aorta [21] is not inconsistent with the longitudinally decreasing rupture force detected by our read-out system in the mouse aorta (i.e., the increased wall thickness results in higher rupture force in aortic segments closer to the heart). Aortic wall thickness was not determined in our study as, in our opinion, the rupture force of aortic rings with standardized width is the clinically most relevant read-out parameter directly related to aortic dissections/ruptures and, as our data show, sufficient to reveal significant difference between wild-type and Col3a1-deficient mice. Notably, differences in rupture force among the three tested aortic sections were less pronounced in heterozygous mice (Fig. 1c). This might be explained by the dependence of rupture force on the intramural collagen content, which is reduced in our heterozygous animals and may decrease from the ascending towards the descending aorta [22, 23].

Accordingly, the expected reduced collagen amount of our heterozygous *Col3a1* mice was observed using multiphoton and electron microscopy images, thereby revealing not only reduced collagen content but also larger spaces among collagen fibrils and reduced alignment/orientation of collagen structure. These observations may explain the reduced rupture force of aortic rings in our heterozygous mice. As neither multiphoton nor electron microscopy allows discrimination between different types of collagens, however, it remains to be determined whether the observed differences in rupture force and collagen characteristics solely reflect the reduced amount (true haploinsufficiency) of type III collagen or whether there are also (indirect) effects of other types of collagens (e.g., due to regulation by *Col3a1*). Indeed, variability in diameters of collagen fibrils was also observed in homozygous animals of the first *Col3a1* mouse model (C.129S4(B6)-Col3a1tm1Jae/J), which can be explained by the hypothesis that type III collagen regulates the diameter of type I collagen fibrils still expressed in homozygous *Col3a1* mice [11].

In methodological context, our measurements at tissue rupture resulted in reproducible tensile forces and confidence intervals that enable to detect significant differences between wild types and heterozygotes in both proximal and distal thoracic aortic segments. This high reproducibility of our read-out system may be due to the standardized measurement procedure and, most importantly, to the reproducible cutting of 1.5-mm-width aortic rings using an appropriate device (Supplementary Fig. 1a). The maximum tensile forces determined *ex vivo* by our read-out system, however, might not reflect rupture forces in physiological *in vivo* conditions with long-term pulsatile pressure in arteries, which could lead to a rupture at lower loads. Moreover, the question remains whether the knowledge obtained in *Col3a1* haploinsufficient mice is transferable to humans [24], especially to cases with other mutation types (i.e., without haploinsufficiency).

Nevertheless, despite advances in diagnostic and surgical possibilities, defects in type III collagen still lead to a substantial risk for severe complications and increased mortality due to aortic dissection and rupture, which may result in shortened life expectancy of EDS IV

patients [3, 25, 26]. For the first *Col3a1* mouse model (C.129S4(B6)-Col3a1tm1Jae/J), recently published studies showed that treatment with doxycycline, a non-selective and broad-spectrum MMP inhibitor, leads to an increased mechanical integrity of the aortic wall [13, 14]. Our data demonstrate that doxycycline is also able to increase the resistance of the thoracic aortic wall to rupture force in heterozygotes to the level of wild types in the novel (second) *Col3a1* mouse model (Col3 alpha 1 delta) already after 4 weeks of treatment. This provides the proof of principle that our read-out system is suitable to test the effect of candidate drugs on the mechanical integrity of the thoracic aorta, at least in the used mouse model, opening the way for the identification and development of novel pharmacological therapies that lower the risk for life-threatening aortic dissections and ruptures in EDS IV.

Electronic supplementary material
The online version of this article contains supplementary material, which is available to authorized users.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.
Ethical approval All applicable international, national and institutional guidelines for the care and use of animals were followed.

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Figure legends

Fig. 1 (**a**) Location of the three 1.5-mm-width ring sections measured in the ascending (section S1) and descending (sections S2 and S3) thoracic aorta. Aorta and heart from a 16-week-old male wild-type animal were exemplarily stained with yellow latex as described elsewhere [27]. (**b**) Rupture forces of the three aortic sections from wild-type animals at different ages. Note that rings from the descending part (S2 and S3) of the aorta ruptured at significantly lower forces compared to the ascending part (S1). (**c**) Rupture forces for wild-type (WT) and heterozygous (HET, Col3 alpha 1 delta) mice at different ages. Note significant differences between corresponding heterozygotes and wild types. Error bars indicate 95% confidence intervals, significant differences in rupture forces are noted (*); n=5 age- and genotype-matched male mice per section

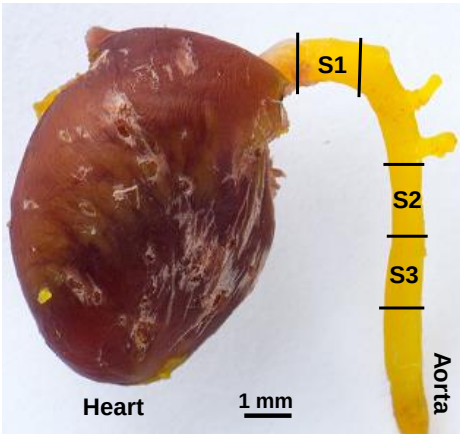
Fig. 2 Transmission electron microscopy images of aorta and skin of 16-week-old wild-type (WT) and heterozygous (HET) mice. (**a**, **b**) Cross sections of collagen fibrils in the adventitia of the thoracic aorta from a heterozygous (**a**) and a wild-type (**b**) mouse. Arrows point to individual fibrils. The collagen fibrils of the tissue from the heterozygous animal are often thicker or thinner and relatively unequal compared to the fibrils of wild-type mouse aorta. (**c**, **d**) Skin section of a heterozygous mouse (**c**) showing less collagen density compared to that of a wild-type mouse (**d**). The arrow in section d indicates longitudinal aligned bundles of collagen fibrils, which are only displayed in the tissue from wild-type animals. Microscope magnification: 24’500×

Fig. 3 Collagen distribution in the aortic wall of 11-week-old wild-type (WT) and heterozygous (HET) mice imaged by multiphoton microscopy. (**a**) 3D images of the whole cross-section of the aortic wall thickness at unstretched configuration showing less densely packed collagen network in the sample of a heterozygous animal compared to a wild-type. (**b**) 2D images made at relaxed configuration and displacements (λ) of 1.9 mm, 2.6 mm, and 3.2 mm. Red arrows indicate stretched collagen structure in the tissue from a wild-type mouse already at a displacement of 1.9 mm. For a heterozygous mouse, the collagen network showed no reaction until a displacement of 3.2 mm

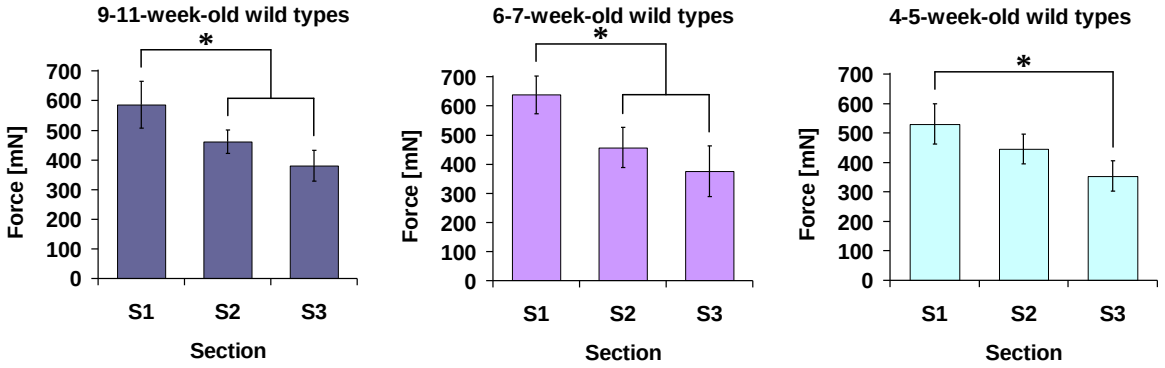
Fig. 4 Effect of doxycycline in the novel *Col3a1* mouse model (Col3 alpha 1 delta). Rupture forces of ring sections of the thoracic aorta (S1-S3, Fig. 1a) of 8-week-old untreated wild-type (WT; n=12, 6 males and 6 females) and heterozygous (HET; n=9, 2 males and 7 females) animals as well as heterozygous mice treated with doxycycline (HET + Doxy; n=9, 2 males and 7 females). Note that in the group treated with doxycycline the rupture force of the aorta was significantly higher compared to untreated heterozygous mice and comparable to the level of wild-type mice. Error bars indicate 95% confidence intervals

Figure 1

a


S1
S2
S3
Aorta
Heart
1 mm

b


9-11-week-old wild types
6-7-week-old wild types
4-5-week-old wild types
Force [mN]
0
100
200
300
400
500
600
700
S1
S2
S3
Section
*

c

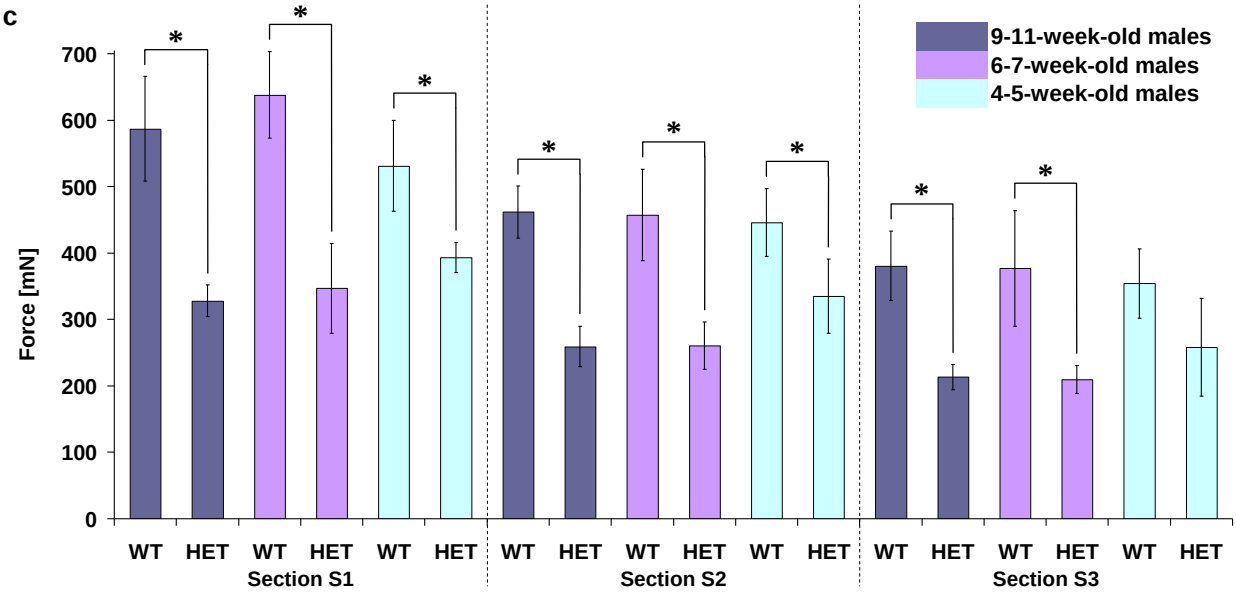

9-11-week-old males
6-7-week-old males
4-5-week-old males
Force [mN]
0
100
200
300
400
500
600
700
WT
HET
Section S1
Section S2
Section S3
*

Figure 2

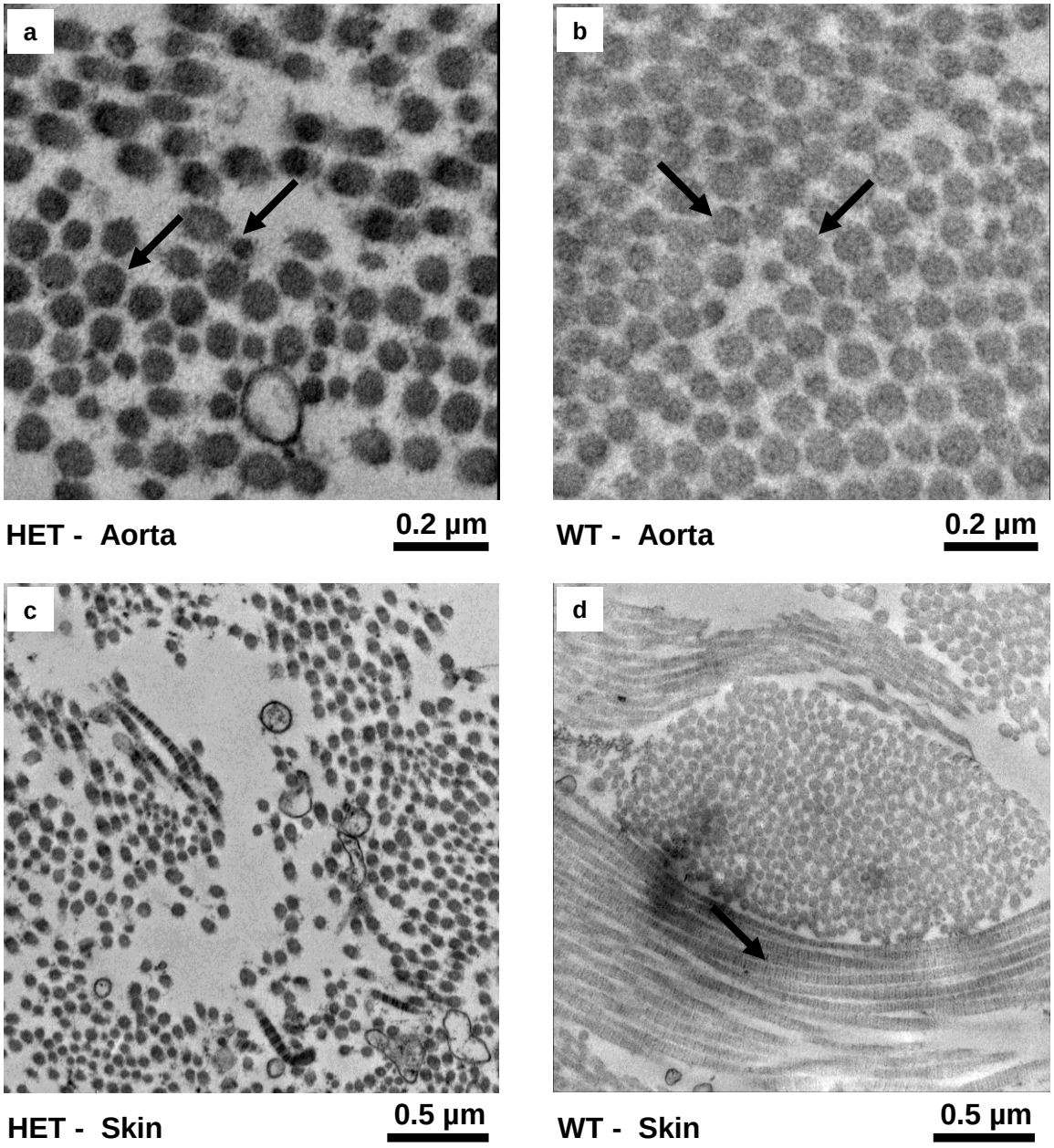


Figure 3

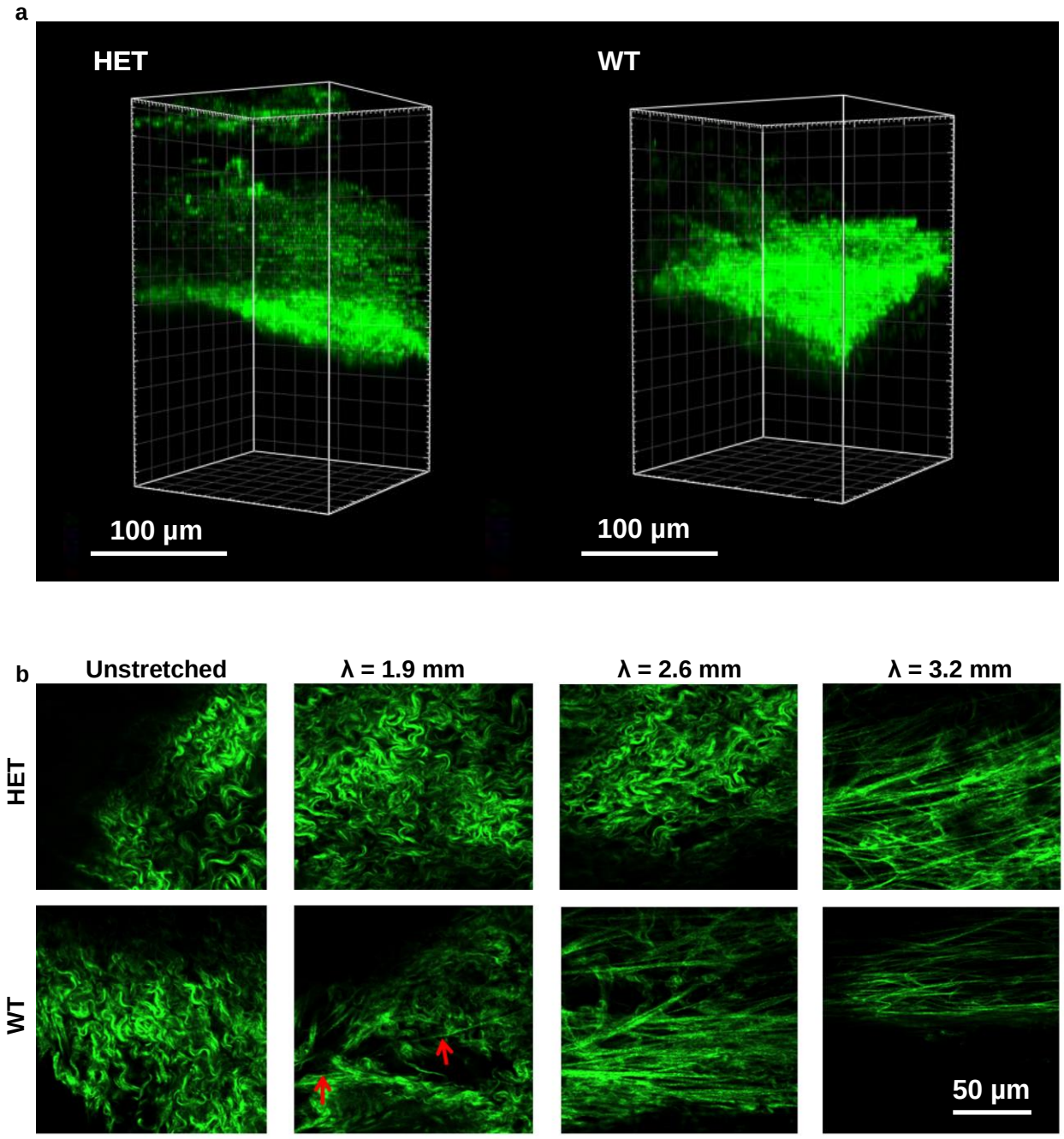
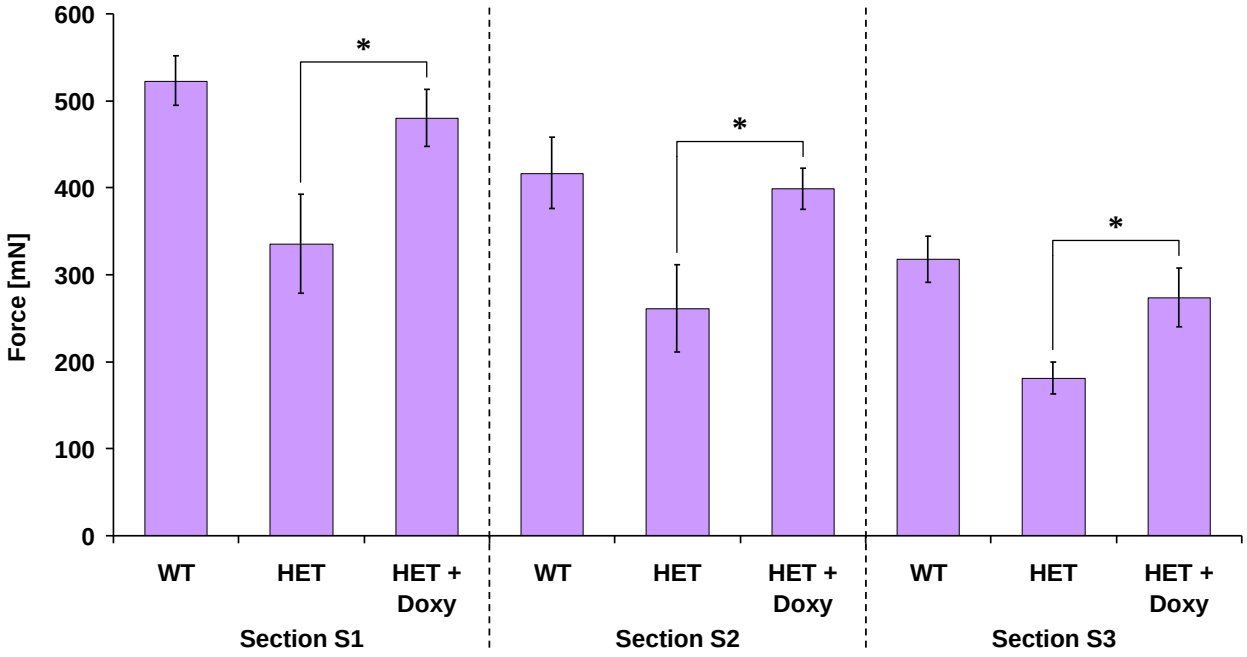

a
HET
WT
100 µm
100 µm
b
Unstretched
λ = 1.9 mm
λ = 2.6 mm
λ = 3.2 mm
HET
WT
50 µm

Figure 4


600
500
400
300
200
100
0
Force [mN]
*
*
*
WT
HET
HET + Doxy
WT
HET
HET + Doxy
WT
HET
HET + Doxy
Section S1
Section S2
Section S3

Cardiovascular Drugs and Therapy – Electronic Supplementary Data

Ehlers-Danlos syndrome vascular type: Mouse model-based read-out system to assess drug effects on the mechanical property of the thoracic aorta

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Table 1 Rupture forces (mN) for 1.5-mm-width ring sections of the thoracic aorta (sections S1-S3, cf. Fig. 1 and Supplementary Fig. 2). Data are mean ± 95% confidence interval, where n=5 per group

	9-11 weeks			6-7 weeks			4-5 weeks		
	Section S1	**Section S2**	**Section S3**	**Section S1**	**Section S2**	**Section S3**	**Section S1**	**Section S2**	**Section S3**
Female WT	657.2±115.6	453.2±110.9	407.0±81.3	637.4±102.0	452.4±84.4	391.4±64.2	no data	no data	no data
Female HET	379.6±110.2	301.2±79.3	265.8±29.4	325.4±51.0	296.6±44.1	265.0±49.0	no data	no data	no data
Male WT	587.2±78.9	462.0±39.3	380.8±52.0	638.2±65.0	457.6±68.8	376.8±87.1	531.2±68.3	446.2±51.1	354.4±52.2
Male HET	328.2±23.6	259.4±30.2	213.4±18.8	347.0±67.7	260.6±35.4	209.8±21.2	393.4±22.4	334.8±55.9	258.4±73.5

WT, wild-type; HET, heterozygous

Table 2 Collagen amount (μm^3) and density of aortic sections of 11-week-old wild-type (WT) and heterozygous (HET) mice calculated at unstretched configuration and at a displacement (λ) of 1.9 mm (cf. Fig. 3). Means and 95% confidence intervals (CI) are noted. In the unstretched configuration, considerable differences in mean collagen amount and density were found between samples of wild-type (M135, M240, M241) and heterozygous (M134, M6, M239) animals

	Sample	**Collagen amount (unstretched) [μm^3]**	**Collagen amount (λ=1.9 mm) [μm^3]**	**Collagen density (unstretched)**
WT	M135	1'493'695	860'823	0.133
	M240	1'231'716	819'286	0.103
	M241	1'605'694	1'130'103	0.160
	Mean	**1'443'702**	**936'737**	**0.132**
	CI	**966'868 – 1'920'535**	**517'524 – 1'355'951**	**0.061 – 0.203**
HET	M134	940'820	151'781	0.077
	M6	1'012'627	1'235'659	0.069
	M239	991'264	992'934	0.063
	Mean	**981'570**	**793'458**	**0.070**
	CI	**889'968 – 1'073'173**	**0 – 2'206'566**	**0.052 – 0.087**

Table 3 Rupture forces (mN) for 1.5-mm-width ring sections of the thoracic aorta (sections S1-S3, see Fig. 1a) showing significant increase in the maximum tensile force of all aortic sections in the group of doxycycline-treated animals compared to heterozygotes fed with control diet (Fig. 4). Data are mean ± 95% confidence interval

	Section S1	**Section S2**	**Section S3**
WT, control diet (n=12)	523.1±28.2	417.1±41.1	318.0±26.7
HET, control diet (n=9)	335.8±56.7	261.6±50.3	181.8±18.3
HET, doxycycline diet (n=9)	480.4±33.2	399.1±23.7	273.9±34.0

WT, wild-type; HET, heterozygous

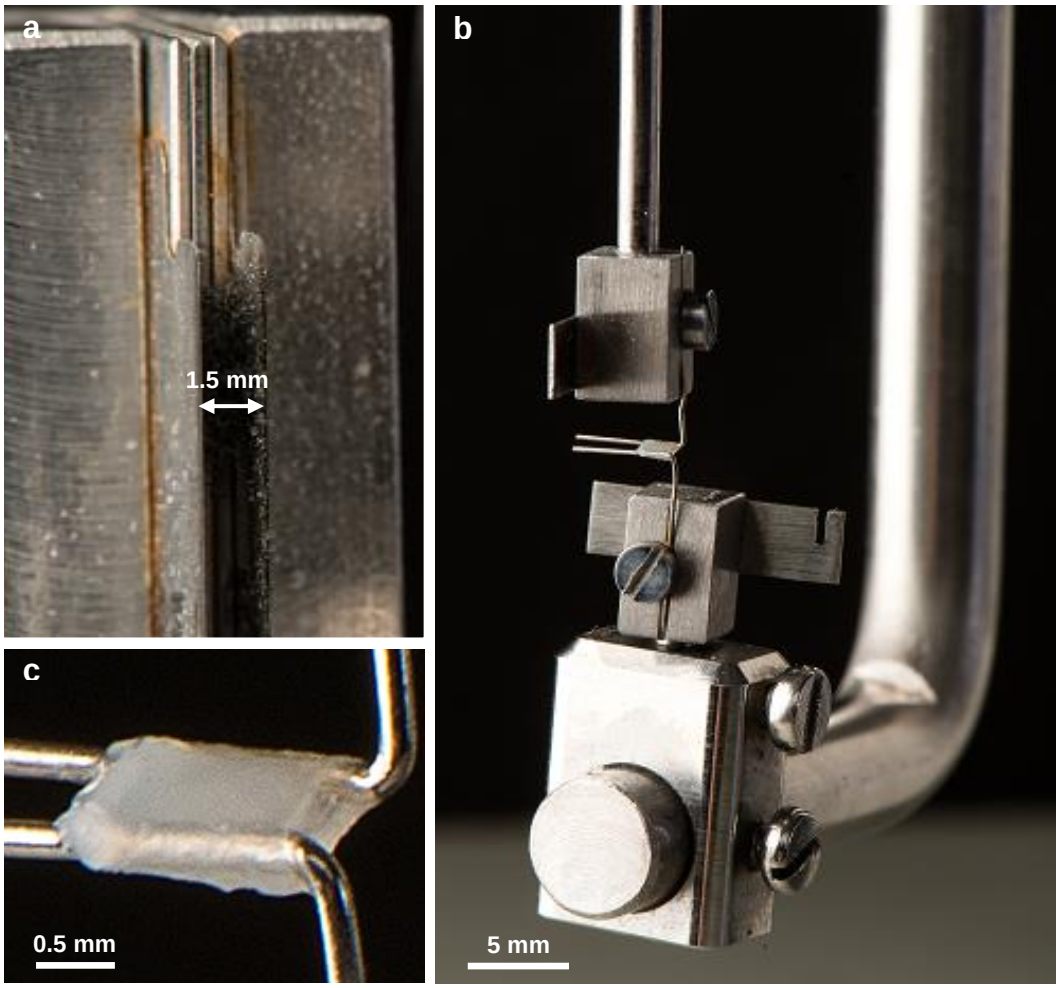

Fig. 1 Set-up for the measurement of tensile force. (**a**) Custom-made device used for cutting 1.5-mm-width aortic rings. (**b**, **c**) Aortic ring mounted on two 200-µm-diameter stainless steel wires of a Tissue Puller 560TP

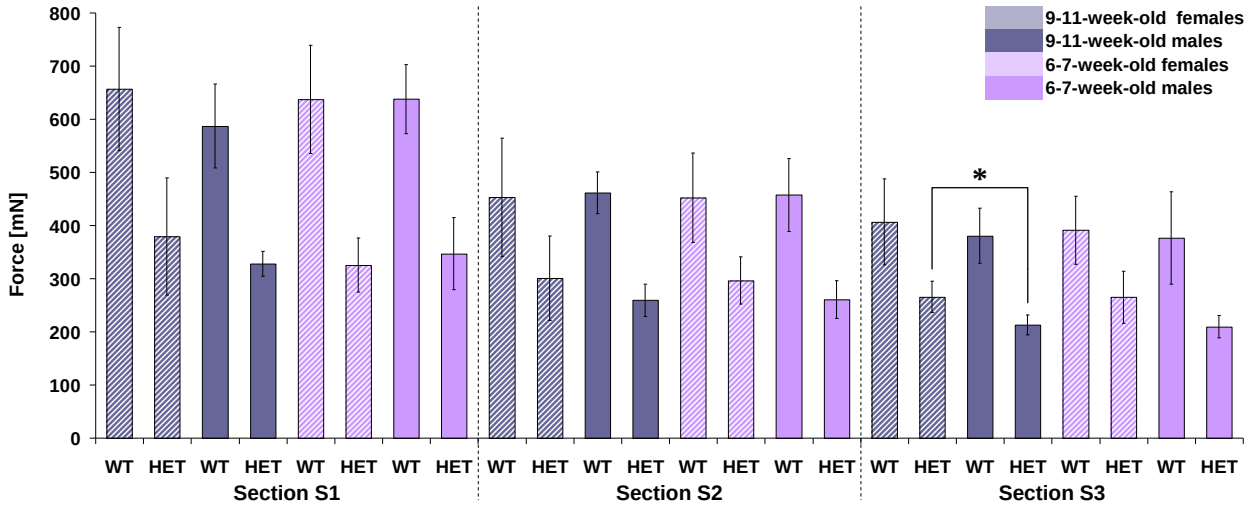

Fig. 2 Rupture forces in female and male animals of the novel *Col3a1* mouse model (Col3 alpha 1 delta). Sections of the thoracic aorta (S1-S3, see Fig. 1a) from wild-type (WT) and heterozygous (HET) mice at different ages (n=5 per group). In all but one case (*), rupture forces showed no significant differences between age- and genotype-matched male and female animals. Error bars indicate 95% confidence intervals

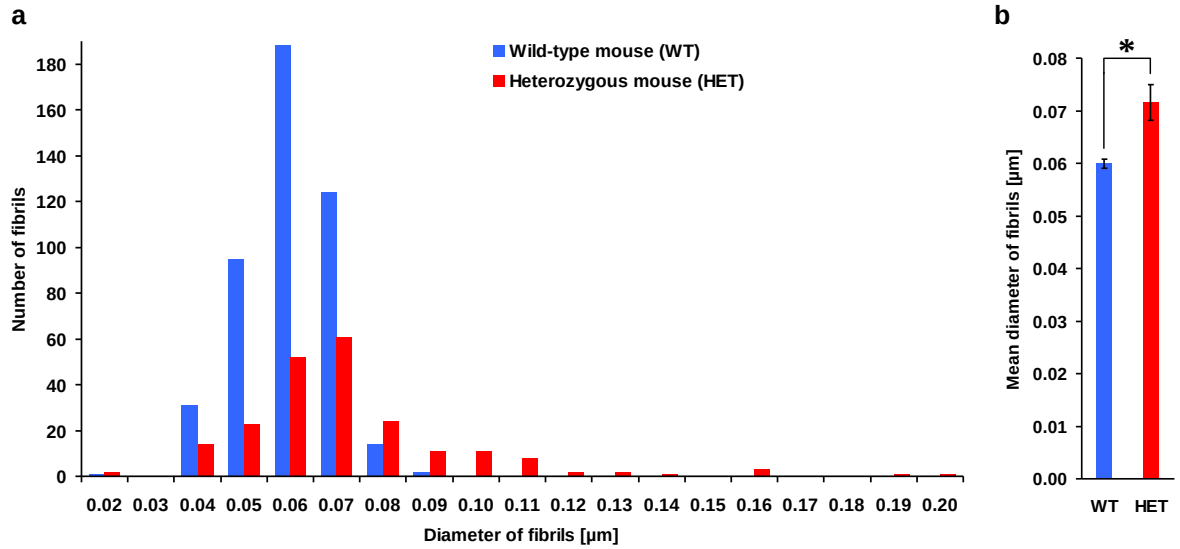

Fig. 3 Collagen fibrils in a 2 µm × 2 µm electron microscopy image area of the adventitia. (**a**) Distribution of fibril diameters for the wild-type (total of 455 fibrils) and the heterozygous mouse (total of 216 fibrils). (**b**) Mean diameter of collagen fibrils showing significant difference between the wild type (WT) and the heterozygote (HET)

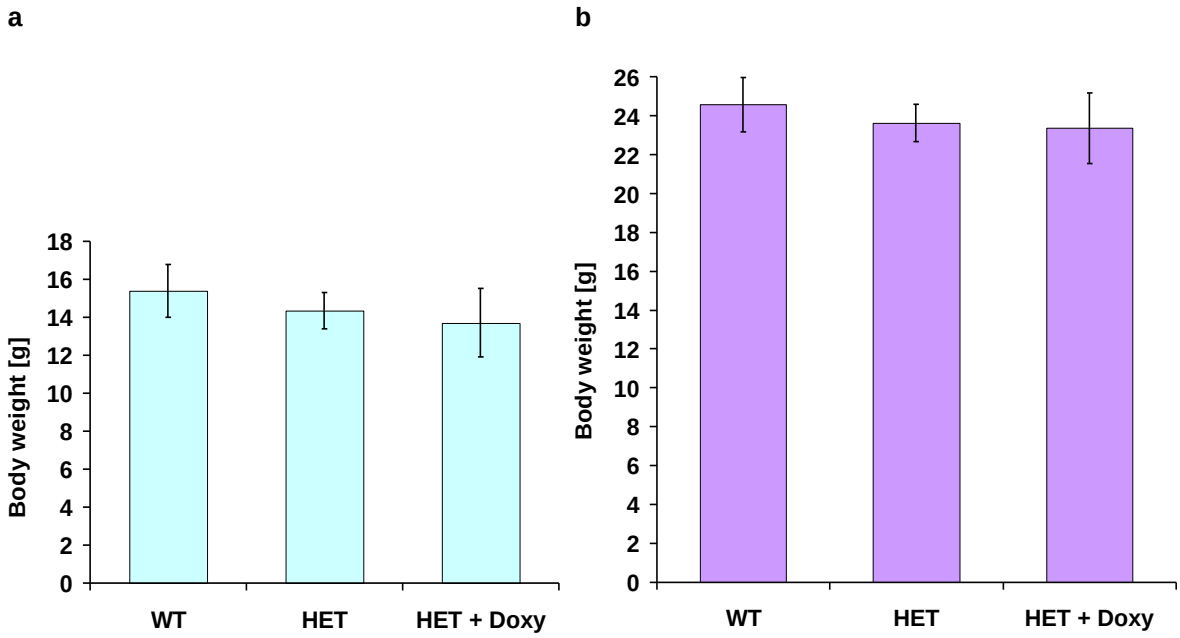

Fig. 4 Mean body weight of animals at the beginning (**a**) and at the end (**b**) of doxycycline treatment showing no differences between wild-type (WT, n=12) and heterozygous mice untreated (HET, n=9) or treated with doxycycline (HET + Doxy, n=9). Error bars indicate 95% confidence intervals

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Justyna Münger	**Conceiving and planning the study, Tissue Puller experiments, electron microscopy, data analysis, writing and editing of the manuscript**
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Jessica Crabb	Stretching experiments and multiphoton microscopy, writing and editing of the manuscript
Erik NTP Bakker	Contribution to the data analysis and editing of the manuscript
Ed van Bavel	Contribution to the data analysis and editing of the manuscript
Urs Ziegler	Electron microscopy, contribution to the data analysis and editing of the manuscript
Thierry Carrel	Contribution to the data analysis and editing of the manuscript
Steffen Zeisberger	Conceiving and planning the study, editing of the manuscript
Gabor Matyas	Initiation of the study, conceiving and planning the study, writing and editing of the manuscript

Danksagung

Mein besonderer Dank gilt Herrn PD Dr. Gabor Matyas für die Gelegenheit an diesem hochinteressanten Thema zu arbeiten und für die Betreuung meiner Arbeit. Ich danke ihm für jede erdenkliche, hilfreiche Unterstützung und viele anregende Diskussionen. Jede Phase dieser Arbeit wurde von ihm intensiv und professionell begleitet.
Herrn Prof. Max Gassmann danke ich für die gewissenhafte Durchsicht und Prüfung dieses Manuskripts und für die Vertretung meiner Dissertation gegenüber der Fakultätsversammlung als Referent.
Bei Frau Prof. Christine Attenhofer Jost bedanke ich mich für die Prüfung dieser Arbeit als Korreferentin.
Frau Dr. Janine Meienberg danke ich für die mir jederzeit gewährte wertvolle Unterstützung bei der Planung, Durchführung und Auswertung der vorliegenden Arbeit.
Allen Mitarbeitern des Zentrums für Kardiovaskuläre Genetik und Gendiagnostik möchte ich ein herzliches Dankeschön sagen für die Unterstützung und die freundliche Aufnahme in ihrem Team.
Ein herzliches Dankeschön an meine Familie, Freunde und alle, die mir mit Beiträgen zu dieser Dissertation behilflich waren.

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